NDRI-PR 83-1
JANUARY 1983

ENHANCEMENT OF CELL ATTACHMENT TO A SUBSTRATE COATED WITH ORAL BACTERIAL ENDOTOXIN BY PLASMA FIBRONECTIN

AD A125314

DTIC
ELECTE
MAR 4 1983
H

M. PORVAZNIK
M. E. COHEN
S. W. BOCKOWSKI
E. J. MUELLER II
M. R. WIRTHLIN JR.

DTIC FILE COPY

NAVAL
DENTAL RESEARCH
INSTITUTE

Naval Medical Research and Development Command
Bethesda, Maryland

83 03 04 014

Best
Available
Copy

NAVAL DENTAL RESEARCH INSTITUTE
NAVAL BASE, BUILDING 1-H
GREAT LAKES, ILLINOIS 60088

ENHANCEMENT OF CELL ATTACHMENT TO A SUBSTRATE COATED WITH ORAL BACTERIAL ENDOTOXIN BY PLASMA FIBRONECTIN

M. PORVAZNIK

M. E. COHEN

S. W. BOCKOWSKI

E. J. MUELLER II

M. R. WIRTHLIN JR.

Research Progress Report NDRI-PR 83-01
Work Unit MR000.01.01-0028
Naval Medical Research and Development Command
National Naval Medical Center
Bethesda, Maryland 20814

The opinions expressed herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or the Naval Service at large. The use of commercially available products does not imply endorsement of these products or preference to other similar products on the market.

This document has been approved for public release; its distribution is unlimited.

Approved and released by:

G. E. CLARK
Captain, DC, USN
Commanding Officer

Journal of Periodontal Research 17: 154–168, 1982

Enhancement of cell attachment to a substrate coated with oral bacterial endotoxin by plasma fibronectin

M. Porvaznik, M. E. Cohen, S. W. Bockowski, E. J. Mueller II and M. R. Wirthlin, Jr.

Naval Dental Research Institute, Great Lakes, Illinois,
Naval Health Research Center, San Diego, California and
Headquarters, Treasure Island, Naval Regional Dental Center, San Francisco, California, U.S.A.

Normal human fibroblasts were inhibited from attaching to a culture surface that was coated with endotoxic lipopolysaccharide (LPS) isolated from oral strains of *Bacteroides* or *Fusobacterium*. The LPS from *Fusobacterium* inhibited cell attachment to a greater degree ($p < 0.001$) than that of the LPS from *Bacteroides*, and at a concentration as low as 12.5 ng/cm² ($p < 0.001$). When LPS-coated culture dishes (12.5 ng/cm²) were incubated with concentrations of plasma fibronectin as low as 50 μg/ml for at least 15 min, the fibronectin significantly enhanced cell attachment, returning it to control levels ($p < 0.001$). Furthermore, when plasma fibronectin (50 μg/ml) was compared to other agents such as bile acids, citrated serum, and human plasma fraction IV_1 (Cohn), which have been shown to affect the toxicity of LPS, fibronectin led to significantly more cell attachment in the presence of LPS than any other treatment ($p < 0.001$). Evidence is presented by means of transmission electron microscopy (TEM) that LPS may bind to fibronectin. Subsequently, the cell surface interacts with the fibronectin-LPS and internalizes it via phagocytosis. This mechanism provides for the clearance of LPS from the culture surface.

(Accepted for publication November 2, 1981)

Introduction

Wound healing and repair of periodontal attachment to teeth following surgical trauma is predictable, and the process has been extensively reviewed (Listgarten 1976). However, in cases where the natural attachment has been destroyed by chronic inflammatory periodontal disease, new attachment to the contaminated root surfaces is unpredictable (World Workshop in Periodontics 1966).

A preliminary characterization of the material eluted from the roots of periodontally diseased teeth indicated the presence of bacterial endotoxin (Fine et al. 1980). The endotoxin was shown to be cementum-bound in otherwise normally appearing cementum (Aleo et al. 1974). The diseased root surfaces were shown *in vitro* to be cytotoxic (Hatfield & Baumhammers 1971) and permitted little or no attachment of human gingival fibroblasts (Aleo, De Renzis & Farber 1975). Clearly, if new attachment of periodontal tissues to a tooth is to be made possible, therapeutic measures must

first be developed to remove, alter, or otherwise inactivate the toxic principle of the bacterial endotoxin. Since the mechanical removal of cementum-bound endotoxin would require extensive root planing (Jones & O'Leary 1978, Fine et al. 1980), the search for other therapeutic measures is warranted. Biochemical preparations have been tried successfully *in vitro* (Wirthlin & Hancock 1980). More recently, in a preliminary report, Terranova and Martin (1981), noted a possible role for attachment proteins in new periodontal attachment. They observed that fibroblasts *in vitro* were stimulated by fibronectin to attach to root surfaces from periodontally involved teeth.

The role of circulating plasma fibronectin has been reviewed in relation to wound healing (Kleinman, Klebe & Martin 1981). Briefly, plasma fibronectin was found to mediate cell attachment at levels of 1-5 μg/ml and was found to be highly chemotactic for fibroblasts (Gauss-Müller et al. 1980). Once in the wound area, fibronectin may promote cell-matrix interactions by stimulating fibroblasts to produce extracellular matrices (Foidart et al. 1980). It has also been shown that fibronectin mediates and promotes phagocytosis of fibronectin-receptor complexes by fibroblasts (Grinnell 1980) and by macrophages (Gudewicz et al. 1980). Fibronectin interacts with a number of macromolecules and with cell surfaces (for a review see Ruoslahti, Engvall & Hayman 1981). These binding site-ligand interactions are complex and indicate a variety of possible functional roles in vivo.

In this report, we have compared in vitro the effectiveness of various biological preparations which may affect fibroblast attachment to a substrate that has been previously coated with endotoxin isolated either from *Fusobacterium nucleatum* or *Bacteroides melaninogenicus* ss. *intermedius*. These bacteria were selected because they have been shown to increase in number relative to the population of bacteria within the gingival crevice with advancing gingival inflammation (Van Palenstein Helderman 1975). They are gram negative anaerobes that have an easily identifiable endotoxin (LPS) in electron microscopic preparations (Selvig, Hofstad & Kristoffersen 1971).

Material and Methods

Cells and Culture Conditions

Detroit 550 cells were purchased from the American Type Culture Collection (ATCC CCL 109, Rockville, MD). Detroit 550 was derived from normal foreskin. It is a diploid human fibroblast-like cell and has a finite life span of approximately 25 serial subcultures. Cultures used in our experiments were from the first through the sixth passages beyond the frozen reference stock, which was already in the tenth or eleventh passage. The culture medium consisted of the following components: minimum essential medium (MEM, Eagle) with non-essential amino acids, 1 mM L-glutamine, 1 mM sodium pyruvate, 0.1% lactalbumin hydrolysate, in Earle's BSS, (90%), fetal bovine serum (10%), antibiotic-free. Cell suspensions were tested for mycoplasma and were determined to be free of such contamination (mycoplasma isolation kit, Flow Labs, Rockville, MD). The cells were maintained at 37 C in an atmosphere of 5% CO_2 – 95% air. Cell stocks were propagated in 75 cm^2 (250 ml) tissue culture flasks (Lux Scientific Corp., Newbury Park, CA). Cells were dissociated from the flasks with trypsin-EDTA (GIBCO Labs, Grand Island, NY).

The 35 × 10 mm, 2 mm grid-type tissue culture dishes (Lux Scientific Corp.) were used in all attachment studies. The medium used in all attachment studies consisted of MEM supplemented with 1 mM L-glutamine and 200 μg of bovine serum albumin (BSA) per ml of MEM medium.

DTIC COPY INSPECTED
Availability Codes
Dist Avail and/or Special
A-20

Oral Bacteria and Growth Conditions

Specimens from human periodontal pockets were collected with an oxygen-free gas-flushed syringe under conditions of anaerobiosis and placed in test tubes containing 1 ml of sterile reduced broth. *Bacteroides melaninogenicus* ss. *intermedius* and *Fusobacterium nucleatum* were isolated from China Blue Reinforced Clostridial agar (Van Palenstein Helderman & Winkler 1975). Pure cultures were maintained in thioglycollate medium or veal infusion broth in an anaerobic chamber at 37 C containing 10% H_2, 10% CO_2, and 80% N_2. Each strain of bacteria was identified by means of gas-liquid chromatography and API 20-A test strips (Analytab Products, Plainview, NY) in accordance with *Bergey's Manual of Determinative Bacteria* (Buchanan & Gibbons 1974) and/or the Virginia Polytechnic Institute's *Anaerobe Laboratory Manual* (Holdeman & Moore 1972). Batch cultures of organisms were prepared by inoculation of 1,500 ml of Lombard-Dowell broth in a cotton-stoppered Ehrlenmeyer flask. The cultures were incubated anaerobically for three to five days in an anaerobic chamber (Coy Laboratory Products, Box 1108, Ann Arbor, Michigan 48106) and the bacteria harvested by centrifugation. Pellets of centrifuged bacteria were resuspended in distilled water and re-centrifuged three times to remove remaining broth medium.

Isolation and Assay of Endotoxic Lipopolysaccharide (LPS)

Endotoxic lipopolysaccharide (LPS) was prepared from oral strains of *B. melaninogenicus* and *F. nucleatum* using the phenol-water procedure (Westphal, Lüderitz & Bister 1952) as followed by Hofstad (1968), for *Bacteroides* and by Kristoffersen and Hofstad 1970, for *Fusobacterium*. The amount of LPS isolated was determined using the Limulus Amebocyte Lysate Pyrostat Test Pack (Worthington Biochemical Corp., Freehold, NJ). Pyrostat is a turbidimetric test, and pyrogen concentrations were quantitated spectrophotometrically. Endotoxin concentrations as low as 0.0625 ng ml were detected reliably.

Preparation of LPS Substratum and LPS "Inactivation Formulae"

LPS suspensions either from *B. melaninogenicus* or from *F. nucleatum* were added to each culture dish at 0.0125–125 ng/cm² and allowed to air dry at 38–40 C for 24 h. When rehydrated with the defined medium for the attachment studies the LPS remained affixed to the culture dish surface.

Human plasma fibronectin was purchased from Biomedical Technologies Incorporated (formerly KOR Biochemicals, Cambridge, MA). The fibronectin was reconstituted with 10 ml of sterile, pyrogen-free water to give a concentration of 100 μg/ml. The fibronectin solutions were sterilized by membrane filtration. This protein solution was added to each tissue culture dish at 5–100 μg/ml and incubated for 5–60 min at 37°C. The culture dishes were subsequently rinsed with pyrogen-free water.

One mmolar solutions of the bile acids taurocholic, glycocholic, and deoxycholic were prepared. LPS-coated plates were incubated with each of these solutions for five min, rinsed with pyrogen-free water, and then treated with either citrated serum (Skarnes & Chedid 1964), 0.15% human plasma fraction IV_1 (Cohn) (Rudbach & Johnson 1961), or human plasma fibronectin. Incubations with citrated serum, 0.15% human plasma fraction IV_1, and human plasma fibronectin were 30 min at 37°C unless otherwise indicated in the figure legends. Following the incubations the culture dishes were rinsed thoroughly in pyrogen-free water.

Cell Attachment Conditions

Confluent cultures of Detroit 550 human

fibroblasts were dissociated from the 75 cm² culture flask using trypsin-EDTA. The dissociated cells were centrifuged and resuspended at 1.4–2.5 × 10^4 cells/ml in a sterile mixing flask containing the attachment medium (MEM supplemented with L-glutamine and 200 μg/ml BSA). Cell counts were taken with a hemocytometer before and after cell plating to monitor the homogeneity of the dissociated cell population. One ml volumes were removed from the mixing flask with an Eppendorf pipetter using sterile tips and transferred to the 35 × 10 mm, grid type culture dishes. These cells were incubated for 90 min under standard conditions. Unattached cells were removed, and the culture dishes were fixed for light and electron microscopy. Exact cell counts were done on three 4 mm² areas for each of four culture dishes per treatment group. Cells were visualized by phase contrast optics using the Zeiss Invertoscope D (Carl Zeiss, Inc., New York, NY) equipped with a photographic camera system.

Transmission Electron Microscopy (TEM)

Samples for TEM were fixed for 1 h at 22 C with Karnovsky's fixative (Karnovsky 1965). Cultures were washed three times in sodium cacodylate containing 0.5 mg $CaCl_2$/ml of buffer, pH ~ 7.4 and stored in buffer overnight at 4 C. The samples were postfixed with 1.33% OsO_4 in 0.067 M collidine buffer at 4°C for 1 h and dehydrated through a graded series of ethanol at 22°C. After the final 100% ethanol change, a thin layer of Epon embedment was added just to cover the culture surface and left uncovered overnight. Fresh plastic was then applied the following morning and incubated for three days at 60°C. After removing the specimens from the oven the Epon embedments were peeled away from the tissue culture dishes (Porvaznik, Johnson & Sheridan 1976). The samples were trimmed and mounted on plastic cylinders (Ladd Research Industries, Inc., Burlington, VT). Thin sections, 60–90 nm, were cut with a diamond knife on an LKB Ultratome III vertical and parallel to the plane of the culture surface, post-stained with uranyl acetate and lead citrate and examined with an RCA EMU III transmission electron microscope at 50 KV.

Negative Staining of LPS

Isolated, freeze-dried LPS from either *F. nucleatum* or *B. melaninogenicus* was suspended in 1% phosphotungstic acid at pH ~ 7.1 to give a concentration of 1 μg ml. A drop of this suspension was placed on a formvar carbon-coated grid and allowed to remain for several minutes. The excess was removed slowly with a piece of filter paper and allowed to air dry. The grids were examined using the TEM.

Statistical Treatment of the Data

Analyses of cell attachment counts were conducted with the aid of Hewlett-Packard HP 9845S desk top computer and either HP (part # 09845–15031) or in-house statistical software. The HP software was used for data manipulation, and for one-way analyses of variance (ANOVA) and least significant difference tests (LSD, Kirk 1968). Dunnett's test for comparing all means with a control (Winer 1962) used the mean square (MS) error computed in the one-way ANOVAs. A two-way factorial ANOVA was computed with an in-house program based upon formulations by Bruning and Kintz (1977).

Results

Growth Characteristics of Detroit 550 Human Fibroblasts

The growth data following cell passage showed an initial lag with recovery and a period of linear growth three to eight days after plating until confluency (Fig. 1). At

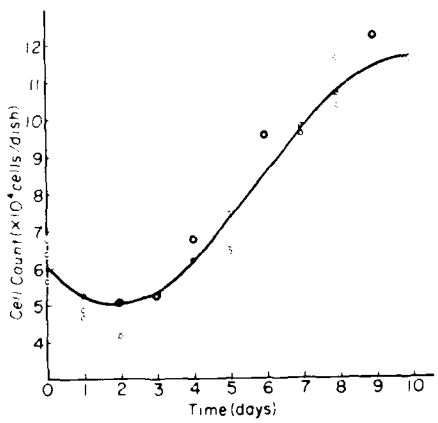


Fig. 1. Typical growth pattern of Detroit 550 human fibroblasts. This type of curve is characteristic for normal contact-inhibited cells. The equation which describes the growth data is the following: $y = 6.08 - 1.36x + 0.45x^2 - 0.03x^3$.

confluency mitotic figures were infrequently observed. A third order polynomial function was fitted to the growth data and the linear, quadratic and cubic trends were found significant (df 1/28; Fs = 305.57, 20.25, and 20.49 respectively) as well as the overall regression ($R^2 = 0.93$; df 3/28; F = 115.44). Thus, these cells behaved in culture like normal contact-inhibited cells and remained in stationary phase at confluency.

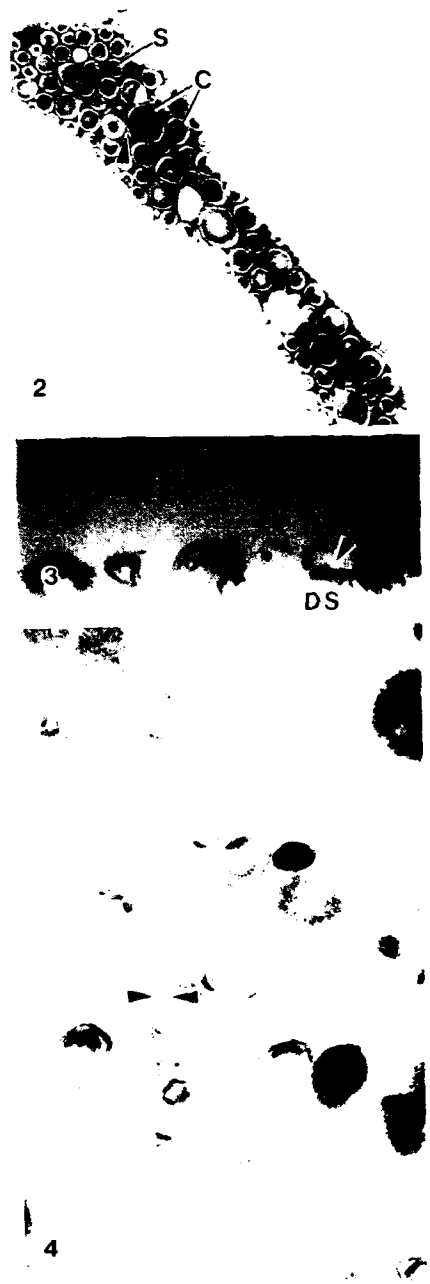


Fig. 2. Transmission electron micrograph (TEM) of a negatively stained isolate of LPS derived from *F. nucleatum*. The predominant forms were translucent disks. Most were single (S) or concentric (C) disks. Occasionally short segments (arrow head) were observed. × 65,200.

Fig. 3. TEM of a thin section (60-90 nm) made perpendicular to the plane of the culture substratum which was 12.5 ng·cm² LPS derived from *F. nucleatum*. The LPS substratum that is displayed lay above the culture dish space (DS). Notice the discoid forms (arrow head) of LPS which were limited by unit membranes. × 66,200.

Fig. 4. TEM of a thin section made parallel to the plane of the LPS substratum. Notice the discoid segments which were a result of an oblique section through the LPS particles. The LPS forms were limited by a unit membrane and were viewed as trilaminar structures (between arrow heads). × 75,300.

Therefore, the cells used for all attachment studies were historically from confluent populations that were not actively dividing. This is important when considering cell surface phenomena, since cells in cycle behave differently and mixed effects would be observed in attachment studies.

LPS Morphology after Isolation and as a Substrate on a Culture Surface

Negatively stained LPS after isolation (Fig. 2) appeared as circular disks limited by a unit or trilaminar membrane or as short parallel membranes separated by a less-dense layer (Figs. 3 & 4). These same structures could be observed in thin sections of intact colonies of *B. melaninogenicus* or *F. nucleatum*. When isolated LPS was coated on a culture surface (12.5 ng/cm²), thin sections made vertical to the plane of culture revealed a layer 0.15–0.25 μm (Fig. 3). The same circular disks and parallel membranes observed in intact colonies and in negatively stained preparations were evident. Thin sections made parallel to the culture surface (Fig. 4) revealed the homogeneity of the LPS coating within a given area on the culture surface. Again, the rehydrated coating of LPS was morphologically similar to freshly isolated LPS.

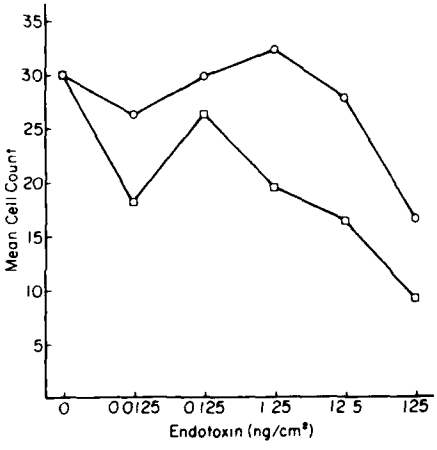


Fig. 5. Each point was the mean cell count of 12 determinations (3 from each of 4 culture dishes). Each determination was an exact count of attached cells in a 4 mm² area on the tissue culture dish. *B. melaninogenicus* LPS-coated culture dishes, ○—○; *F. nucleatum* LPS-coated culture dishes □—□.

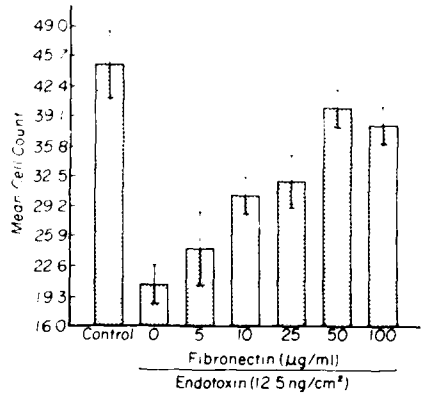


Fig. 6. Histogram of the mean cell count of attached cells ± S.E.M. on culture dishes coated with 12.5 ng cm² of LPS from *F. nucleatum*. LPS-coated dishes were pretreated with plasma fibronectin (5 100 μg ml) for 30 min at 37 C prior to the 90 min incubation with 1.88 × 10⁴ fibroblasts/culture dish.

Attachment of Human Fibroblasts to LPS-Coated Culture Surfaces

The relative toxicity of isolated LPS from *B. melaninogenicus* or *F. nucleatum* that inhibited fibroblast attachment to a culture surface coated with the specific LPS was compared (Fig. 5). Data were subjected to a two-way between subjects ANOVA with endotoxin type (*B. melaninogenicus* vs. *F. nucleatum*) and concentration (6 levels: 0, 0.0125, 0.125, 1.25, 12.5, and 125 ng/cm²) being the factors. To simplify the analysis to that of a complete factorial design, the 12 data points collected with zero level endotoxin were incorporated into both endotoxin conditions. Both toxin type ($F = 13.93$, $df = 1/132$, $p<0.001$) and concentration ($F = 6.65$, $df = 5/132$, $p<0.001$) were significant, but the interaction was not ($F = 1.02$),

Using Dunnett's method for comparing groups to a control, concentration levels of 0.0125 ng·cm² and above were compared to the zero level within each endotoxin type at $\alpha = 0.05$. For *B. melaninogenicus* only the 125 ng·cm² group differed from the control but for *F. nucleatum* both the 12.5 and the 125 ng·cm² groups differed. The *F. nucleatum* LPS inhibited fibroblast cell attachment to a pretreated substrate surface at a concentration one-tenth that of the *B. melaninogenicus* LPS (12.5 and 125 ng·cm², respectively). It should be noted that in this and subsequent analysis each of the three observations within a single culture dish were considered independent. In order to evaluate this assumption, individual one-way ANOVA's were undertaken on the control group and each of the ten endotoxin groups with "culture dish" considered a treatment effect. None of the F values were significant at $\alpha = 0.05$ (df = 3/8). The average F value was 1.11, and the average p value was 0.52. The average MS between treatments was 120.14, and the average MS within treatments was 131.31. Thus, there is no evidence that variability within culture dishes was less than variability between culture dishes, and the assumption that each observation was independent is valid.

Because there was a significant difference in cell attachment from the control at 12.5 ng/cm² for *F. nucleatum* LPS-coated cul-

Fig. 7. Phase contrast light micrograph 90 min post-incubation in a control (untreated) culture. Several fibroblasts (arrow heads) have attached and were in the process of cell spreading. × 750.

Fig. 8. Phase contrast light micrograph 90 min post-incubation on an LPS substratum (LPS, 12.5 ng·cm²) derived from *F. nucleatum*. The paucity of attached cells (arrow heads) was apparent. Those cells present were typically round and not well spread. × 750.

Fig. 9. Phase contrast light micrograph 90 min post-incubation on an LPS substratum (LPS, 12.5 ng·cm²) derived from *F. nucleatum* that was treated with 50 μg/ml of plasma fibronectin for 30 min prior to cell incubation. Notice the fully spread cells (arrow heads) compared to the other preparations. × 750.

ture surfaces and not for *B. melaninogenicus*, that concentration and source of LPS was used on all subsequent culture surface coatings as a minimum inhibitory concentration of endotoxin.

Effect of Fibronectin Concentration and Time of Incubation on LPS-Coated Culture Dishes

In Fig. 6 each of five concentrations of fibronectin was incubated for 30 min on the culture dishes previously coated with 12.5 ng/cm² of LPS derived from *F. nucleatum*. Cell attachment was evaluated on the treated culture dishes after 90 min in culture.

The one-way ANOVA on data from the control, LPS only, and 5 LPS plus fibronectin concentration groups was significant ($F = 8.52$, $df = 6/77$, $p<0.001$). Comparisons by Dunnett's method indicated that toxin groups treated with 50 to 100 μg/ml of fibronectin were not significantly different from untreated controls while all other LPS groups showed a significant decrease in cell attachment.

The morphology of attached cells on the control surface (Fig. 7), LPS (Fig. 8), and LPS plus fibronectin treated surfaces (Fig. 9) were considerably different at the end of the 90 min attachment period. Although control cells appeared to be spread out on the culture dish, fibronectin treated surfaces enabled the fibroblasts to appear to be spread even further. Within these cells, microfilaments were prominent under the plasma membrane. See Figs. 14, 16, and 17. Cells attached on the LPS-coated dishes were generally rounded without any or very little spreading of the cytoplasm (Fig. 8).

In another study (Fig. 10), the concentration of fibronectin (50 μg/ml) remained constant in all incubations on the LPS-coated culture dishes. The time of incubation was varied from five min to one h. Cell attachment returned to control levels

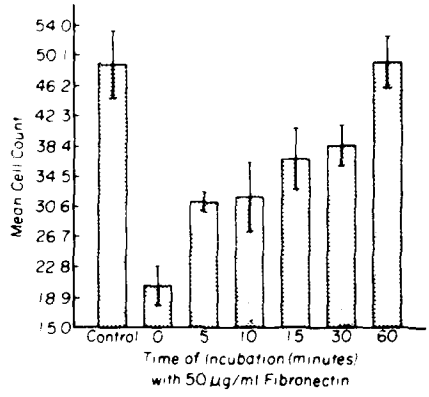


Fig. 10. Histogram of mean cell count of attached cells ± S.E.M. on culture dishes coated with 12.5 ng cm² of LPS from *F. nucleatum*. LPS-coated culture dishes were pretreated with 50 μg ml plasma fibronectin for 5 60 min at 37 C prior to the 90 min incubation with 1.88×10^4 fibroblasts culture dish

on the LPS-coated culture dishes when they were incubated with plasma fibronectin for at least 15 min.

The one-way ANOVA on data from the control, LPS (12.5 ng cm² *F. nucleatum*) only (these two cell groups were part of the previous study, see Fig. 6), and five LPS plus fibronectin incubation time groups was significant ($F = 8.30$, $df = 6/77$, $p<0.001$). Comparisons by Dunnett's method indicated that LPS groups treated with fibronectin (50 μg/ml) for 15, 30, or 60 min were not significantly different from untreated controls, while the 0, 5, and 10 min incubation groups showed significantly reduced numbers of cells attached to the LPS substratum.

LPS and Drug Treatment Interactions which Affect Cell Attachment

The data from comparative drug studies were divided into single drug treatment and dual drug treatment groupings and were analyzed separately. A two-way ANOVA on the seven control and single drug groups versus presence or absence of endotoxin revealed a main effect for drug treatment

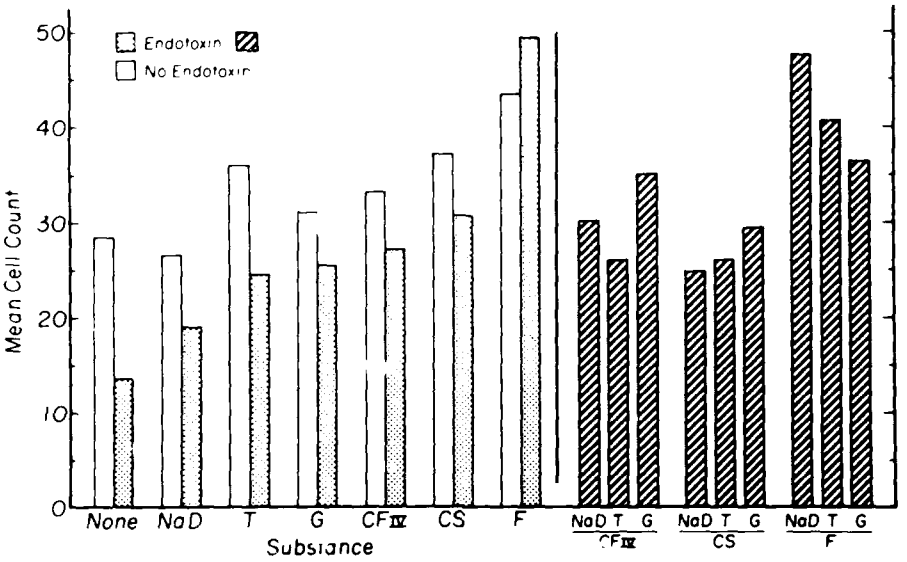


Fig. 11. Histogram of the mean cell count of attached cells. Culture dishes were either coated or uncoated with 12.5 ng mm^2 of LPS from *E. coli* 0.55. LPS-coated and uncoated dishes were treated for 5 min each with either one mmolar sodium deoxycholate (NaD), one mmolar taurocholic acid (T) or one mmolar glycocholic acid (G) prior to cell incubation. One mmolar solutions of the bile acids were used since it was determined that this concentration was not toxic to the human fibroblasts. Alternatively, LPS-coated or uncoated dishes were treated for 30 min with either 0.15% human plasma fraction IV$_1$ (Cohn) (CF$_{IV}$), human citrated serum (CS), or 50 μg ml human plasma fibronectin (F). The percentage of CF$_{IV}$ was the maximum permissible concentration that could be filtered through a 0.22 μm filter without occluding the pores. Additionally, LPS-coated dishes were pretreated with a combination of bile acid for 5 min, rinsed in pyrogen free water, incubated for 30 min at 37 C either with CF$_{IV}$, CS, or F and rinsed again thoroughly with pyrogen free water prior to the 90 min incubation with 1.4×10^4 fibroblasts culture dish.

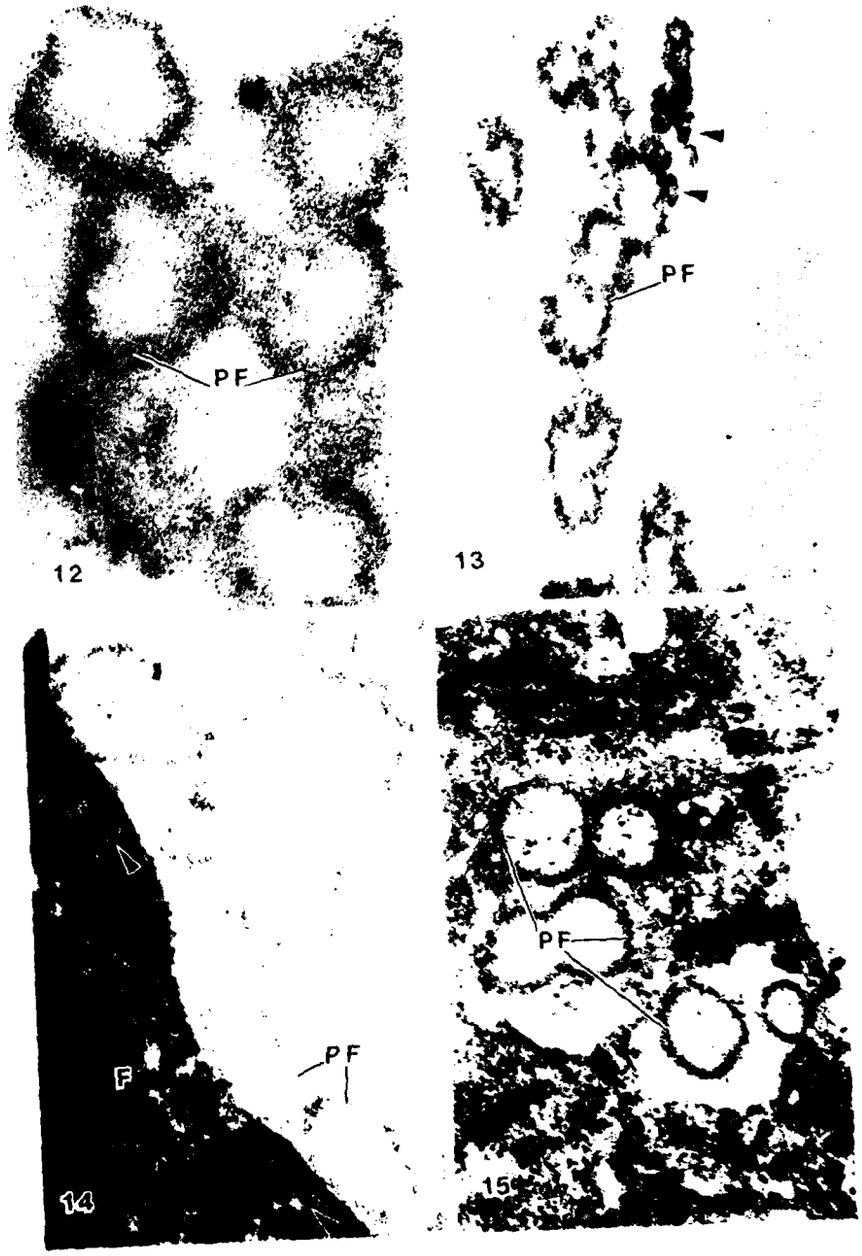
(F = 18.58, df = 6/154, $p<0.001$) and endotoxin (F = 17.51, df = 1/154, $p<0.001$) and a significant interaction (F = 2.88, df = 6/154, $p<0.05$). Inspection of Fig. 11 suggests that this interaction stemmed from the fact that in the presence of fibronectin with endotoxin there appeared to be enhanced cell attachment. Comparisons by Dunnett's method were done independently for non toxin and endotoxin groups. For non-toxin

Fig. 12. High magnification TEM of the presumptive plasma fibronectin (PF) arrays that were observed when PF was incubated on a culture substrate. Notice the ring-like array of the otherwise amorphous material. Sectioned parallel to the culture surface. × 91,000

Fig. 13. TEM of a presumptive plasma fibronectin array (PF) apparently attached to LPS particles (Arrow heads). Sectioned parallel to the culture surface. × 38,000

Fig. 14. TEM of a presumptive plasma fibronectin array (PF apparently attached to the plasma membrane of a human fibroblast (F). Notice the microfilaments underlying the cell surface adjacent to the fibronectin attachment (arrow heads). Sectioned parallel to the culture surface. × 39,400

Fig. 15. TEM showing the ring-like arrays of the presumptive fibronectin (PF) within the cytoplasm of a fibroblast. Sectioned parallel to the culture surface. × 38,000


PF
12
PF
13
F
PF
14
PF
15

groups only fibronectin enhanced cell attachment; however, in the endotoxin groups only sodium deoxycholate did not enhance cell attachment. Analysis in terms of the LSD statistic revealed among other contrasts that fibronectin led to significantly better cell attachment than any other treatment in the presence of endotoxin and was matched only by taurocholic acid and citrated serum in its absence. The superiority of fibronectin is supported by the second analysis on the paired drug treatments. A two-way ANOVA was performed where the first three-leveled factor was determined by the presence of sodium deoxycholate, taurocholic acid, or glycocholic acid and the second factor by the presence of human plasma fraction IV_1 (Cohn), citrated serum, or fibronectin. Only the treatments which included incubation with bile acid followed by fibronectin were significant ($F = 18.81$, $df = 2, 99$, $p > 0.001$), and inspection of the figure again indicates the comparative potency of this drug. The interaction was also significant ($F = 2.59$, $df = 4, 99$, $p < 0.05$) and suggests that fibronectin interacts with sodium deoxycholate, taurocholic acid, and glycocholic acid differently than human plasma fraction IV_1 (Cohn) and citrated serum.

Electron Microscopic Identification of LPS and Drug Treatment Interactions with Dextran 550 Human Fibroblasts

When examined by TEM, fibronectin treated culture plates showed many amorphous ring-like arrays in the plane of cell culture (Fig. 12). They were present when fibronectin was incubated on LPS-coated culture dishes (Fig. 13) but were not present either on control or on LPS-coated culture dishes. Fibroblasts appeared to be attached to the material (Fig. 14). LPS-coated culture dishes that were incubated with fibronectin showed no apparent alteration in the morphology of the LPS.

Examination by TEM of citrated serum and human plasma fraction IV_1 (Cohn) treated culture dishes with or without LPS also showed these amorphous ringlike arrays but were infrequently observed. Furthermore, examination by TEM of the LPS on the culture plates treated with bile acids or followed by citrated serum, human plasma fraction IV_1 (Cohn), or fibronectin showed no obvious differences in the morphology of the LPS compared to untreated LPS controls.

The presumptive plasma fibronectin observed by TEM on the culture surface was also observed in contact with apparent LPS particles (see Fig. 13). The presumptive fibronectin arrays and LPS particles were observed within phagocytic vacuoles inside the human fibroblasts (Figs. 15 & 16, respectively). Fragments of these arrays and apparent LPS particles were also observed within phagocytic vacuoles (Fig. 17), although not as clearly as the extracellular view presented in Fig. 13. A biological mechanism for the clearance of LPS from the culture surface was depicted by these micrographs.

Discussion

Human fibroblasts were inhibited *in vitro* from attaching to culture dishes coated with endotoxic lipopolysaccharide (LPS) derived from oral strains of *B. melaninogenicus* or *F. nucleatum*. LPS from the latter strain was significantly more effective in preventing cell attachment when compared to LPS from *B. melaninogenicus* ($p < 0.001$). Based upon inhibition of cell attachment, there appeared to be a tenfold difference in the biological toxicity of the endotoxin types examined. The level of toxicity on a substrate surface, which was observed to be inhibitory, was at least 12.5 ng of LPS/cm^2.

One of us (Mueller, unpublished results) has independently shown that compared to

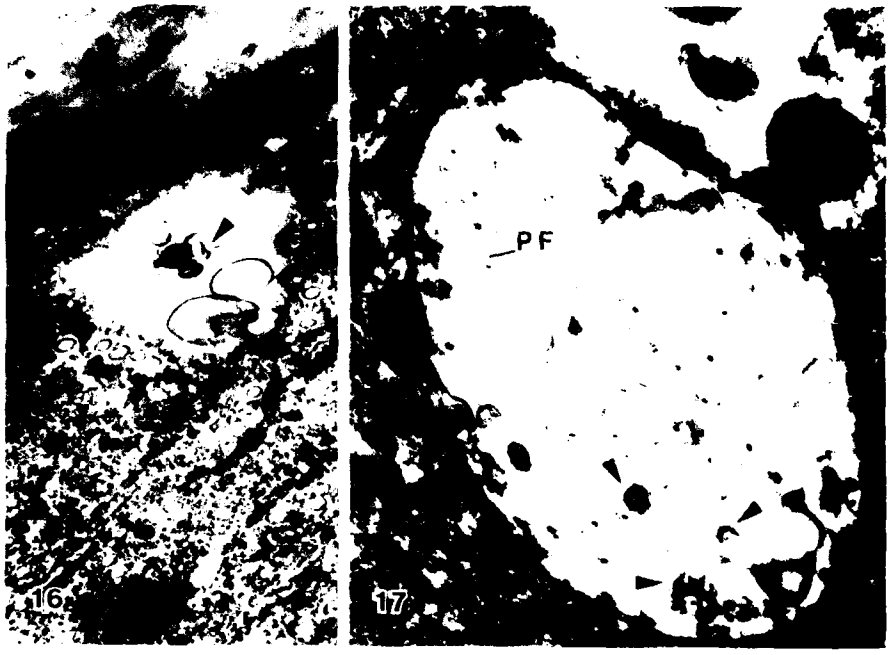


Fig. 16. TEM showing a fibroblast that appeared to have internalized some LPS particles (arrow head). Sectioned parallel to the culture surface. × 28,800.

Fig. 17. TEM of a large phagocytic vacuole within a fibroblast. The digestive vacuole contained fragments of fibronectin (PF) and LPS particles (arrow heads) that appeared to be attached to each other. Sectioned parallel to the culture surface. × 26,200.

Bacteroides, *Fusobacterium* may promote greater bone loss in rice rats that have been put on a diet high in sucrose and whose dentition and gingivae were swabbed periodically with the individual microbes. This has led us to suggest that different Gram negative anaerobes and/or their toxins may have more detrimental effects in the course of periodontal disease and may affect the success of new attachment therapy *in vivo*.

An important requirement of new attachment therapy and for wound healing in general is close adaptation of the gingival tissues to the tooth at the completion of surgery (Schluger, Yuodelis & Page 1977). It is believed that intimate contact favors a shorter epithelial attachment (Morris 1961) and is thought to result in early formation of an epithelial seal, protecting the connective tissue from bacterial irritants which could interfere with cementogenesis (Ruben et al. 1980).

Since oral bacterial endotoxins are present on diseased root surfaces and in dental plaque matrix (Selvig et al. 1971), close adaptation may be inhibited thereby preventing new connective tissue attachment (Aleo et al. 1975). Methods must first be developed to degrade, alter, or modify the endotoxic LPS present on the root surfaces.

The physical preparation of scaling and vigorous root planing reduces the endotoxin level (Jones & O'Leary 1978) but may create a problem of root hypersensitivity for the

patient (Wirthlin & Hancock 1980). Other methods for the biochemical detoxification of endotoxin contamination have involved a biological preparation using bile salt (Wirthlin & Hancock 1980), human plasma fraction IV_1 (Cohn) (Wirthlin & Hancock 1980), citrated serum (Skarnes & Chedid 1964), or fibronectin (Terranova & Martin 1981). These preparations apparently affected the endotoxic LPS in some fashion permitting greater cell attachment to the treated surface.

We have examined similar preparations using these treatments to obviate the inhibition of cell attachment that we observed on endotoxic LPS coated culture dishes. Our results revealed that fibronectin led to significantly better cell attachment than any other treatment in the presence of LPS. TEM did not reveal any direct effect of the biochemical treatment on the morphology of the LPS. However, subtle chemical changes in LPS may have occurred but were not detected using TEM. Alternatively, it is known that fibronectin is identical to opsonic α_2-glycoprotein and has been responsible for enhancing phagocytosis, suggesting that plasma fibronectin may serve as a non-specific opsonin (Blumenstock et al. 1978). If indeed, plasma fibronectin is an opsonin, LPS may bind to the fibronectin and be presented to the fibroblasts through binding sites for cell surfaces (Ruoslahti et al. 1981). Once this interaction occurs, coated LPS may be internalized and digested within the cell's lysosomal system. We have shown by TEM evidence which supports this hypothesis.

This method could facilitate the elimination of such obviously harmful particles from the root surface making it a more attractive site for tissue healing by connective tissue new attachment. However, the application times used in these experiments may be too long to be clinically useful during surgical flap procedures. Wirthlin and Hancock (1981) found that rubbing each axial root surface of periodontally involved teeth from monkeys with NaD for one min, then each with CF_{IV} for one min, was a very long time altogether. Longer times would likely increase trauma to the soft tissue flaps and bone. When using fibronectin as has been done in this report, it would not be necessary to rub the root surface but only bathe it. However, these methods would be more suitable where extracted teeth may be prepared in vitro before replantation. Fibronectin may also be used for preparing non-tooth, allopastic implant materials. Concentrations and application methods must be developed with the idea of using them in surgical procedures.

Acknowledgements

We wish to thank HM1 Eric W. Larson and DT1 John M. McCormick for the graphic illustrations. We also thank Ms. Myra J. Rouse for typing and editing the manuscript.

The opinions expressed herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or the naval service at large. The use of commercially available products does not imply endorsement of these products or preference to other similar products on the market.

Supported by Naval Medical Research and Development Command work unit MR 000.01.01–0028.

References

Aleo, J. J., De Renzis, F. A., Farber, P. A. & Varboncoeur, A. P. 1974. The presence of biologic activity of cementum-bound endotoxin. *Journal of Periodontology* **45**: 672–675.

Aleo, J. J., De Renzis, F. A. & Farber, P. A. 1975. *In vitro* attachment of human gingival fibroblasts to root surfaces. *Journal of Periodontology* **46**: 639–645.

Blumenstock, F. A., Saba, T. M., Weber, P. & Laffin, R. 1978. Biochemical and immunological characterization of human opsonic α_2-SB glycoprotein: Its identity with cold-insoluble globulin. *Journal of Biological Chemistry* **253**: 4287–4291.

Bruning, J. L. & Kintz, B. L. 1977. *Computational Handbook of Statistics*, 2nd ed. Glenview, Illinois: Scott Foresman and Co.

Buchanan, R. E. & Gibbons, N. E. 1974. *Bergey's Manual of Determinative Bacteriology*, 8th edition. Baltimore, Maryland: Williams and Wilkins Co.

Fine, D. H., Morris, M. L., Tabak, L. & Cole, J. D. 1980. Preliminary characterization of material eluted from the roots of periodontally diseased teeth. *Journal of Periodontal Research* **15**: 10–19.

Foidart, J. M., Berman, J. J., Paglia, L., Rennord, S. L., Abe, S., Perantoni, A. & Martin, G. R. 1980. Synthesis of fibronectin laminin and several collagens by a liver-derived epithelial cell line. *Laboratory Investigation* **44**: 525–532.

Gauss-Müller, V., Kleinman, H. K., Martin, G. R. & Schiffman, E. 1980. Role of attachment and attractants in fibroblast chemotaxis. *Journal of Laboratory and Clinical Medicine* **96**: 1071–1080.

Grinnell, F. 1980. Fibroblast receptor for cell-substratum adhesion: Studies on the interaction of baby hamster kidney cells with latex beads coated by cold insoluble globulin (plasma fibronectin). *Journal of Cell Biology* **86**: 104–112.

Gudewicz, P. W., Molnar, J., Zong Lai, M., Beeghold, D. W., Siefring, G. E. Jr., Credo, R. B. & Lorand, L. 1980. Fibronectin-mediated uptake of gelatin-coated latex particles by peritoneal macrophages. *Journal of Cell Biology* **87**: 427–433.

Hatfield, C. G. & Baumhammers, A. 1971. Cytotoxic effects of periodontally involved surfaces of human teeth. *Archives of Oral Biology* **16**: 465–468.

Hofstad, T. 1968. Chemical characteristics of *Bacteroides melaninogenicus* endotoxin. *Archives of Oral Biology* **13**: 1149–1155.

Holdeman, L. V. & Moore, W. E. C. 1972. *Anaerobe Laboratory Manual*. Blacksburg, Virginia: Virginia Polytechnic Institute.

Jones, W. A. & O'Leary, T. J. 1978. The effectiveness of *in vivo* root planing in removing bacterial endotoxin from the roots of periodontally involved teeth. *Journal of Periodontology* **49**: 337–342.

Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology* **27**: 137a (abstract).

Kirk, R. E. 1968. *Experimental Design: Procedures for the Behavioral Sciences*. Belmont, California: Brooks/Cole.

Kleinman, H. K., Klebe, R. J. & Martin, G. R. 1981. Role of collagenous matrices in the adhesion and growth of cells. *Journal of Cell Biology* **88**: 473–485.

Kristoffersen, T. & Hofstad, T. 1970. Chemical composition of lipopolysaccharide endotoxins from human oral Fusobacteria. *Archives of Oral Biology* **15**: 909–916.

Listgarten, M. 1976. Ultrastructural features of repair following periodontal surgery. In: *Periodontal Surgery*, ed. Stahl, S. S., pp. 189–207. Springfield, Illinois: Charles C. Thomas.

Morris, M. L. 1961. Healing of naturally occurring periodontal pockets: Preliminary report of the effects of external pressure. *Journal of Periodontology* **32**: 202.

Porvaznik, M., Johnson, R. G. & Sheridan, J. D. 1976. Intracellular junctions and other cell surface differentiations of H4-IIE hepatoma cells *in vitro*. *Journal of Ultrastructure Research* **55**: 343–359.

Ruben, M. P., Smuckler, H., Schulman, S. M., Kon, S. & Bloom, A. A. 1980. Healing of periodontal surgical wounds. In: *Periodontal Therapy*, ed. Goldman, H. M. & Cohen, D. W., p 640. St. Louis: C. V. Mosby.

Rudbach, J. A. & Johnson, A. G. 1961. Alteration of bacterial endotoxin by human plasma fraction IV_1 (Cohn). *Bacteriological Proceedings* p 131 (M 117).

Ruoslahti, E., Engvall, E. & Hayman, E. G. 1981. Fibronectin: Current concepts of its structure and functions. *Collagen Research* **1**: 95–128.

Schluger, S., Yuodelis, R. A. & Page, R. C. 1977. *Periodontal Disease*, p 483. Philadelphia: Lea and Febiger.

Selvig, K. A., Hofstad, T. & Kristoffersen, T. 1971. Electron microscopic demonstration of bacterial lipopolysaccharides in dental plaque matrix. *Scandinavian Journal of Dental Research* **79**: 409–421.

Skarnes, R. C. & Chedid, L. C. 1964. Biological degradation and inactivation of endotoxin. In: *Bacterial Endotoxins*, ed. Landy, M. & Braun, W. New Brunswick, New Jersey: Institute of Microbiology, Rutgers, The State University.

Terranova, V. P. & Martin, G. R. 1981. A possible role for attachment proteins in periodontal reattachment. *Journal of Dental Research* **60**: 9 (abstract 320).

Van Palenstein Helderman, W. H. & Winkler, K. C. 1975. Elective medium for the direct count of vibrio (campylobacter), fusobacteria, bacteroides, selenomonas, and veillonella in the gingival crevice flora. *Journal of Periodontal Research* **10**: 230–231.

Van Palenstein Helderman, W. H. 1975. Total viable count and differential count of vibrio (campylobacter) sputorum, fusobacterium nucleatum, selenomonas sputigena, bacteroides ochraceus, and veillonella in the inflamed and non-inflamed human gingival crevice. *Journal of Periodontal Research* **10**: 294–305.

Westphal, O., Luderitz, O. & Bister, F. 1952. Über die extraktion von bakterien mit phenol /gasser. *Zeitschrift Naturforsch* **7B**: 148–155.

Winer, B. J. 1962. *Statistical Principles in Experimental Design*. New York: McGraw-Hill.

Wirthlin, M. R. & Hancock, E. B. 1980. Biologic preparation of diseased root surfaces. *Journal of Periodontology* **51**: 291–297.

Wirthlin, M. R. & Hancock, E. B. 1981. Regeneration after biologic treatment of root surfaces in monkeys. *Journal of Dental Research* **60**: 336 (abstract 101).

World Workshop in Periodontics, p. 324. 1966. Ann Arbor: University of Michigan Press.

Address:

Naval Dental Research Institute
Naval Base, Bldg. 1-H
Great Lakes, Illinois 60088
U. S. A.

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A125314	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) ENHANCEMENT OF CELL ATTACHMENT TO A SUBSTRATE COATED WITH ORAL BACTERIAL ENDOTOXIN BY PLASMA FIBRONECTIN	5. TYPE OF REPORT & PERIOD COVERED
	6. PERFORMING ORG. REPORT NUMBER NDRI-PR 83-01
7. AUTHOR(s) M. PORVAZNIK, M. E. COHEN, S. W. BOCKOWSKI, E. J. MUELLER II and M. R. WIRTHLIN JR.	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Naval Dental Research Institute Naval Base, Building 1-H Great Lakes, Illinois 60088	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS MR000.01.01-0028
11. CONTROLLING OFFICE NAME AND ADDRESS Naval Medical Research and Development Command National Naval Medical Center Bethesda, Maryland 20814	12. REPORT DATE JANUARY 1983
	13. NUMBER OF PAGES 15
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Naval Medical Command Navy Department Washington, D. C. 20372	15. SECURITY CLASS. (of this report) UNCLASSIFIED
	15a. DECLASSIFICATION DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

This document has been approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

This document has been approved for public release; distribution unlimited.

18. SUPPLEMENTARY NOTES

Journal of Periodontal Research 17:154-168, 1982

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Plasma fibronectin
Cell culture
Attachment
Fibronectin
Endotoxin
Periodontal disease

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Normal human fibroblasts were inhibited from attaching to a culture surface that was coated with endotoxic lipopolysaccharide (LPS) isolated from oral strains of Bacteroides or Fusobacterium. The LPS from Fusobacterium inhibited cell attachment to a greater degree ($p<0.001$) than that of the LPS from Bacteroides, and at a concentration as low as 12.5 ng/cm^2 ($p<0.001$). When LPS-coated culture dishes (12.5 ng/cm^2) were incubated with concentrations of plasma fibronectin as low as 50 µg/ml for at least 15 min, the fibronectin significantly enhanced cell attachment, returning it to control levels

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE
S-N 0102-LF-014-6601

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

($p<0.001$). Furthermore, when plasma fibronectin (50 μg/ml) was compared to other agents such as bile acids, citrated serum, and human plasma fraction IV_1 (Cohn), which have been shown to affect the toxicity of LPS, fibronectin led to significantly more cell attachment in the presence of LPS than any other treatment ($p<0.001$). Evidence is presented by means of transmission electron microscopy (TEM) that LPS may bind to fibronectin. Subsequently, the cell surface interacts with the fibronectin-LPS and internalizes it via phagocytosis. This mechanism provides for the clearance of LPS from the culture surface.